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(54) Method for the preparation of a protein hydrolyzate from whey protein.

(57) Disclosed is a process for the preparation of a protein hydrolyzate suitable for use in an enteric diet. The method involves the enzymatic hydrolysis of whey protein (particularly lactalbumin) using foodgrade fungal protease produced by an organism of the species *Aspergillus oryzae*.

**EP 0 065 663 A1**

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1        METHOD FOR THE PREPARATION OF A  
         PROTEIN HYDROLYZATE FROM WHEY PROTEIN

BACKGROUND OF THE INVENTION

         Enteric diets, i. e., those diets which comprise  
5    nutrients which are designed to pass through the stomach un-  
         altered and be absorbed by the intestines, are necessitated by  
         various disorders. A suitable enteric material must, of  
         course, contain amino acids to provide complete nutrition.

         It does not seem necessary that an elemental enteric  
10   diet contain only amino acids. Rather, recent evidence sug-  
         gests that peptides 2-3 units long are absorbed even more  
         readily than the individual amino acids in some cases. The  
         original theory of protein absorption was that small peptides  
         liberated by the pancreatic proteases were hydrolyzed to their  
15   constituent amino acids by brush border peptidases. These  
         amino acids are then transported across the cell membrane  
         by an active transport system coupled to the sodium pump.  
         It now appears that in addition to this there is a mechanism  
         specific for the uptake of small peptides. Di- and tri-  
20   peptides are actively transported against concentration  
         gradients by a common mechanism and are later hydrolyzed by

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- 1 cytoplasmic peptidases. Longer peptides are hydrolyzed at  
the brush border membrane with absorption of the resultant  
amino acids or di- to tri- peptides. The rates of absorption  
of peptides are frequently faster than those of the free amino  
5 acids, and the peptides are absorbed well in the proximal and  
distal small intestine, while free amino acids are absorbed  
most readily in the proximal region.

There is also evidence that peptide diets may be useful  
in the treatment of amino acid absorption diseases. In  
10 Hartnupp's disease, transport of neutral amino acids is di-  
minished but the transport of peptides is unaffected. In  
cystinuria, small intestinal absorption of cysteine, ornithine,  
arginine and lysine are diminished but again the absorption of  
amino acids is normal if they are administered as di- and tri-  
15 peptides. In Lowe's syndrome, there is a decrease in all  
amino acid transport although peptide transport is not affected.  
In fact, there is no evidence of a primary peptidase deficiency  
or a peptide transport deficiency analogous to that of amino  
acids.

- 20 Malabsorption of amino acids is associated with many  
small intestinal disorders whereas dipeptide absorption is less  
severely affected. In tropical and celiac sprue, absorption  
of free amino acids is reduced while dipeptide absorption is  
fairly normal. In jejunioleostomy for obesity, a reduction of  
25 the absorption of free amino acid leucine occurs without any  
reduction in the absorption of the dipeptide glycyl-leucine.

Diets of free amino acids are hyperosmotic as compared  
to peptide diets and it has been shown that a peptide diet causes  
less fluid secretion into the small intestine than an equal  
30 nitrogen content amino acid diet.

1           In view of the above discussion, it is apparent that an  
elemental enteric diet containing amino acids and peptides  
would be preferable to one containing only amino acids. Op-  
timally the peptides should contain from 2 to 3 amino acid  
5   residue groups. While the optimal 2 to 3 group peptide may  
not be achievable on a commercial scale at a reasonable cost,  
a material containing a substantial amount of di- and tri-  
peptides along with some amino acids and higher molecular  
weight polypeptides is suitable for use in an enteric diet. The  
10   di- and tri- peptides would be transported intact, whereas, the  
tetra-, penta and hexa- peptides would be hydrolyzed by pri-  
mary brush border peptidases and the resulting di- and tri-  
peptides transported across the cell membrane. Amino acids  
would be absorbed in some cases and excreted in others, de-  
15   pending on the malady involved. Higher molecular weight  
polypeptides would be excreted. Thus, a protein hydrolyzate  
suitable for use in an enteric diet will desirably contain at  
least 50 weight percent of a combination of amino acids, di-  
peptides and tri- peptides and not more than 25 weight percent  
20   of polypeptides containing 10 or more amino acids;

Amino acids and peptides used in elemental enteric  
diets may be prepared by the enzymatic hydrolysis of a pro-  
tein source material. In view of the above discussion, it can  
be seen that control of the molecular weight distribution of a  
25   protein hydrolyzate for use in such a diet is essential.

The flavor of its protein hydrolyzate is also a major  
factor in the success of an elemental enteric diet. Thus, it  
is desirable not only to produce a protein hydrolyzate with the  
proper molecular weight distribution but also one with a bland  
30   flavor. The protein source can have a major effect on the

1 flavor of a hydrolyzate produced from it. Legumes such as  
soybeans are notorious for their bitter, grassy, burnt, catty  
and fusel notes. Many of the compounds responsible for these  
flavors (long chain alcohols, ketones and aldehydes) are com-  
5 pounds of the raw bean and decrease on heating, but new ones  
develop. Further processing is necessary to remove these  
materials.

Fish protein concentrates also present problems.

Fish protein is usually contaminated with 1°, 2° and 3° amines  
10 which contribute significantly to its characteristic flavor. In  
addition, fish muscle contains from 1-16% fat which is poly-  
unsaturated. These lipids are difficult to extract and are  
readily oxidized by air or lipooxygenases and esterases (present  
in the fish flesh) to objectionably flavored compounds. The  
15 protein hydrolyzate itself presents a flavor problem since  
many amino acids, especially the more hydrophobic ones, are  
themselves bitter. Although the particular enzyme applied in  
the hydrolysis exerts some effect on the level of bitterness,  
the protein itself is also a factor.

20 It is also required that the protein hydrolyzate have a  
protein efficiency ratio (PER) at least equal to that of whole  
egg, i. e., at least 2.5. For this reason, and because of its  
bland flavor, we prefer to use whey protein (PER of 3.0) as  
the starting material for our hydrolyzate. In the production  
25 of cheese, milk solid, i. e., casein, is precipitated from milk  
either by acid precipitation or enzymatic coagulation leaving  
a liquid phase containing whey proteins. The solid whey pro-  
tein can be recovered by various techniques such as heat pre-  
cipitation, reverse osmosis, gel filtration and electrodialysis.  
30 We prefer to use a whey protein obtained by heat precipitation  
(lactalbumin).

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1           Lactose, which is a contaminant of most whey pro-  
teins is not digested by a large proportion of the population  
and is a common cause of gastrointestinal complaints. There-  
fore, it must be present only in very low levels, if at all, in  
5   the hydrolyzate. The amount of lactose that produces symp-  
toms has been investigated, and based on the investigations it  
can be concluded that whey protein used as the starting ma-  
terial for a protein hydrolyzate should contain a level of lactose  
such that the hydrolyzate produced from it will contain no  
10   greater than 1.0 weight percent of the sugar.

#### SUMMARY OF THE INVENTION

The present invention is a method for the preparation  
of a protein hydrolyzate suitable, both in terms of molecular  
weight profile and flavor, for use in products to be consumed  
15   by individuals on an enteric diet. The method comprises the  
steps of: .

- a) providing whey protein having a lactose level suf-  
ficiently low to provide a protein hydrolyzate containing no  
more than 1.0 weight percent of this sugar;
- 20       b) forming an aqueous slurry of the whey protein;
- c) adding foodgrade neutral fungal protease from  
Aspergillus oryzae to the slurry in an amount of from about  
18.9 to 189 spectrophotometric hemoglobin units per gram of  
whey protein;
- 25       d) maintaining the pH and temperature of the slurry  
containing the protease at a level of from about pH 3.0 to about  
10.0 and from about 40° C. to about 70° C. for a time suffi-  
cient to hydrolyze the whey protein into a hydrolyzate con-  
taining at least 50 weight percent of a combination of amino

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- 1 acids, di-peptides and tri-peptides and not more than 25  
weight percent of polypeptides containing 10 or more amino  
acids;
- 5 e) heating the slurry to a temperature and for a time  
sufficient to inactivate the enzyme;
- f) removing remaining solid material from the slurry  
to provide an aqueous solution containing the desired protein  
hydrolyzate; and
- 10 g) recovering the protein hydrolyzate from the  
solution.

#### DETAILED DESCRIPTION

The first step of this method involves the procurement of low lactose whey protein. Since we prefer to use lactalbumin as the protein source, the following discussion  
15 will be directed to the use of this particular whey protein. The lactose level of lactalbumin can be minimized by washing the sugar from the heat precipitated curd either before or after it is dried. When lactalbumin prepared in this manner is unavailable, lactose can be removed by hydrolysis with lactase.

20 Experiments involving the formation of hydrolyzates from enzymatically hydrolyzed lactalbumin were carried out using the following enzymes:

1. Fungal protease; obtained by controlled fermentation of Aspergillus oryzae var. The enzyme preparation con-  
25 tains a mixture of acid neutral and alkaline proteases exhibiting activity from pH 3.0 to 10.0 but having a maximum at pH 9.0. The preparation exhibits proteolytic activity to 70° C., but has a maximum at 55° C. In the examples, we used an enzyme preparation -- Takamine Brand Fungal Protease -- from Miles
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- 1 Laboratories, Inc. which had an activity of 3,780 Spectro-  
photometric Hemoglobin Units (SHU) per gram. One SHU is  
that activity which will liberate one micromole of tyrosine per  
minute under the conditions of the assay according to the ap-  
5 proved methods of the American Association of Cereal  
Chemists, 1969; Proteolytic Activity - Spectrophotometric  
Method (AACC method 22-63); American Association of Cereal  
Chemists, St. Paul, Minnesota.

2. Bacterial protease; obtained by controlled fermenta-  
10 tion of Bacillus licheniformis var. The enzyme preparation  
contains primarily endopeptidases and exhibits proteolytic  
activity from pH 3.0 to 9.0 with a maximum from 5.0 to 5.5.  
The enzyme preparation is active up to 70° C., with a max-  
imum at 55° C. We used an enzyme preparation -- Alcalase  
15 0.6L -- from Novo Industri A/S, Bagsvaerd, Denmark, having  
an activity of 628 SHU/gm.

3. HT Proteolytic concentrate, Papain 3,000 and Pan-  
creatin 4NE, all produced by Miles Laboratories, Inc., were  
also tested under their optimal conditions.

- 20 All of the resulting protein hydrolyzates were evaluated  
for flavor, molecular weight distribution and amino acid pro-  
file. The fungal protease concentrate was judged as the pre-  
ferred material for the production of lactalbumin hydrolyzate  
to be used in enteric diets. The other enzyme preparations  
25 failed primarily due to unpleasant product flavor and less de-  
sirable peptide size of the protein hydrolyzate.



1        Although the fungal protease concentrate was found to  
be the best protease preparation for lactalbumin hydrolysis  
in respect to flavor and peptide size, one drawback in using  
this enzyme was found to be a low hydrolyzate yield of about  
5    40%. To increase the accessibility of substrate to enzyme,  
the lactalbumin slurry was subjected to acid (30 minutes  
boiling in 2%  $H_2SO_4$  solution) or alkali (10 minutes boiling at  
pH 8.0) prior to enzymatic hydrolysis. The results of this  
experiment are set out in Table II.

10 TABLE II  
Effect of Heat Treatment on Hydrolysis Yield

	Lactalbumin Treatment	Enzyme Level (% w/w)	Hydrolysis Conditions	Yield (%)	ApL*
15	Control	1.0	pH 7.0, 50° C, 7 hr.	39.6	2.3
	Acid-Heating	1.0	pH 7.0, 50° C, 7 hr.	28.8	3.2
20	Alkali-Heating	1.0	pH 7.0, 50° C, 7 hr.	58.2	2.4

\* Average peptide length

The average peptide length is the ratio of amino nitrogen to total nitrogen. Amino nitrogen was determined by trinitro-  
25 benzene sulfonic acid (TNBS) according to the description of Adler Nissen (J. Ag. Food Chem., 27:1256, 1979). Total nitrogen was determined by the Kjeldahl method:

1 From Table II it can be determined that the alkali-heat  
treatment of lactalbumin rendered the protein more accessible  
to protease attack, thereby increasing the yield, without sig-  
nificantly affecting the average peptide length of the hydroly-  
5 zate.

Lactalbumin was screened into different particle sizes  
(50, 100 and 200 mesh) after being treated with alkali and  
hydrolyzed with 1% fungal protease concentrate for 7 hours  
at pH 7.0 and 50° C. The results are set out in Table III.

10

TABLE III  
Effect of Particle Size on Hydrolysis Yield

	Particle Size (Mesh)	Yield (%)
15	50	57.3
	100	58.7
	200	55.8

From Table III it can be determined that particle size of  
lactalbumin does not significantly affect the degree of  
20 hydrolysis.

Sensory evaluation of lactalbumin hydrolyzates prepared  
with various enzymes showed that 1% fungal protease or 1%  
fungal proetase combined with 1% Alcalase yielded the product  
having the least flavor. Hydrolyzates prepared with 10%  
25 Alcalase were extremely bitter, especially when digested under  
alkali conditions. Two percent (2%) Pancreatin 4NE also pro-  
duced bitter hydrolyzates, intermediate between fungal

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1 protease and Alcalase. Ten percent (10%) Alcalase or a  
combination of 1% fungal protease with 10% Alcalase was ex-  
tremely bitter. Miles Laboratories, Inc. HT Proteolytic  
concentrate and Papain 3,000 also produced a bitter hydroly-  
5 zate. Depending on the various reaction conditions such as  
temperature, pH and protease concentration, the hydrolysis  
is typically carried out for a period of 2 to 50 hours.

Using 1% fungal protease, hydrolyzates were prepared  
with different antimicrobial agents (200 ppm sulfite or 1%  
10 toluene). Neither of these samples were significantly dif-  
ferent in flavor from the control. During the alkaline heating  
step and the hydrolysis reaction, a base is added for pH con-  
trol. We prefer to use a base other than NaOH in order to  
keep the sodium content low.  $\text{Ca}(\text{OH})_2$  is the preferred base  
15 and no difference in flavor was noted when either NaOH or  
 $\text{Ca}(\text{OH})_2$  was used for pH control.

The method of practicing the method is further illus-  
trated by the following example:

#### EXAMPLE I

20 Lactalbumin (35 Kg obtained from New Zealand Dairy)  
was combined with 180 gallons of deionized water in a 200  
gallon kettle equipped with a steam jacket to form a 5% (w/v)  
aqueous slurry. Approximately 18 liters of a 4% NaOH solution  
was added to raise the pH to 7.0 whereupon the temperature  
25 was increased to 60° C. and the slurry stirred for 15 minutes.  
The resultant was centrifuged using a Westfalia Separator (bowl  
speed 6500 RPM, Model 9AMRCO36) to provide 60 gallons of  
sludge and 130 gallons of supernatant containing about 0.5%  
solids which was discarded. An additional 130 gallons of

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- 1 deionized water was added to the sludge to form a 5% (w/v)  
aqueous slurry which was adjusted to pH 7.0, stirred for 15  
minutes at 60° C., centrifuged and separated as before to  
provide 60 gallons of lactalbumin sludge having a lactose  
5 level below 1.0 (w/w) based on the dry lactalbumin. An ad-  
ditional 30 gallons of deionized water was added to provide  
slurry containing about 10% (w/v) solids.

- The lactalbumin slurry was adjusted to pH 8.0 by the  
addition of 4.6 liters of a 4% NaOH solution whereupon the  
10 slurry was heated to 90-95° C. for 10 minutes with stirring  
and then cooled to 50° C. With the pH of the slurry controlled  
at 7.0 by the addition of approximately 200 grams of lime in a  
10% slurry, 300 grams (3780 SHU/gm.) of Miles fungal pro-  
tease dissolved in 10 liters of water was added to the slurry.
- 15 The slurry was maintained at 50° C. for 24 hours whereupon  
it was heated to 90° C. for 5 minutes, then cooled to 50° C.  
The cooled slurry may be clarified by either centrifugation or  
filtration with a commercially available filter aid. The clari-  
fied hydrolyzate may be mixed with the remaining ingredients  
20 of the enteric diet (i. e., carbohydrates, fat, vitamins and  
minerals) and then dried or dried first and then blended with  
the remaining ingredients. A hydrolyzate prepared according  
to the instructions of this example should have an average  
peptide length of 2.3. Lactose content of the lactalbumin hy-  
25 drolyzate was measured using a Lactose Assay Kit from  
Boehringer Mannheim (Indianapolis, Indiana). In it, lactose  
is hydrolyzed to glucose and  $\beta$ -galactose in the presence of  
 $\beta$ -galactosidase and water.  $\beta$ -galactose is oxidized by  
nicotinamide adenine dinucleotide (NAD) to galactonic acid in  
30 the presence of the enzyme  $\beta$ -galactose dehydrogenase and

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- 1 NAD is converted to NADH. The amount of NADH formed is stoichiometric with the amount of lactose. The increase in NADH is measured by means of its absorption at 334, 340 or 365 nm. The lactose level of the hydrolyzate prepared in this example was less than 0.5% (w/w) which means that the formulated diet will contain less than about 0.05% lactose.

#### EXAMPLE II

- The procedure of Example I was repeated except that after 7 hours of digestion with 1% fungal protease an additional 1% of enzyme was added and the digestion continued for an additional 7 hours to provide a protein hydrolyzate having an average peptide length of 2.3.

#### EXAMPLE III

- The procedure of Example II was repeated except that 1% Alcalase was added after 7 hours to provide a protein hydrolyzate having an average peptide length of 2.3.

#### EXAMPLE IV

- The molecular weight profiles of the hydrolyzates prepared in Examples I and II were determined by exclusion chromatography according to the method of Carnegie, Nature, 206:1128, 1965. The gel selected for this was Sephadex G-25, particle size 20-80 $\mu$ . Column dimensions were 90 x 1.5 cm. and the solvent was phenol:acetic acid:water, 1:1:1, wt.:wt.:vol. A calibration curve was determined and elution volumes were calculated for amino acids to peptides of three units long, peptides from 4 to 9 amino acids long and peptides of 10 or

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- 1 more amino acids. Samples of hydrolyzate from Examples  
I and II were evaluated for molecular weight profile by de-  
termining the percent nitrogen present in those three fractions  
of the column eluent. This method, which has a margin of  
5 error of  $\pm 3\%$  indicated that the molecular weight profile of  
the samples analyzed was 65% amino acids and di- and tri-  
peptides; 20% polypeptides of 4 to 9 amino acids and 16% of  
polypeptides containing 10 or more amino acids.

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1. A process for preparing a protein hydrolyzate suitable for use in products to be consumed by individuals on an enteric diet, which process comprises the steps of:

- a) providing whey protein having a lactose level sufficiently low to provide a protein hydrolyzate containing no more than 1.0 weight percent of this sugar;
- b) forming an aqueous slurry of the whey protein;
- c) adding foodgrade neutral fungal protease from Aspergillus oryzae to the slurry in an amount of from about 18.9 to 189 Spectrophotometric Hemoglobin Units per gram of whey protein;
- d) maintaining the pH and temperature of the slurry containing the protease at a level of from about pH 3.0 to about 10.0 and from about 40° C. to about 70° C. for a time sufficient to convert the whey protein into a hydrolyzate containing at least 50 weight percent of a combination of amino acids, di-peptides and tri-peptides and not more than 25 weight percent of polypeptides containing 10 or more amino acids;
- e) heating the slurry to a temperature and for a time sufficient to inactivate the enzyme;
- f) removing remaining solid material from the slurry to provide an aqueous solution containing the desired protein hydrolyzate; and
- g) recovering the protein hydrolyzate from the solution.

2. The process of Claim 1 wherein the whey protein is lactalbumin.

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1           3. The process of Claim 1 wherein the pH is about 9.0.

          4. The process of Claim 1 wherein the temperature is  
about 55° C.

5           5. The process of Claim 1 wherein in addition to the  
fungal protease there is added to the slurry a bacterial pro-  
tease obtained by the controlled fermentation of Bacillus  
licheniformis and the pH is maintained at a level of from 3.0  
to 9.0.

          6. The process of Claim 2 wherein the lactalbumin is  
10 heated in an alkaline solution prior to the enzymatic hydrolysis.

          7. The process of Claim 1 wherein the enzymatic  
hydrolysis is carried out for a period of from 2 to 50 hours.

          8. The process of Claim 1 wherein the pH of the slurry  
is controlled by the use of  $\text{Ca}(\text{OH})_2$ .





European Patent  
Office

# EUROPEAN SEARCH REPORT

0065663

Application number

EP 82 10 3656

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. <sup>3</sup> )
X	LU-A- 40 694 (HOWARD LLOYD)  * Claims 1,2,5; example 1 *	1,2,4,7	A 23 J 3/00
A	US-A-3 761 353 (F.F. NOE, W.T. FAITH) * Claims 1-3 *  -----	1,5	
			TECHNICAL FIELDS SEARCHED (Int. Cl. <sup>3</sup> )
			A 23 J
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 31-08-1982	Examiner PEETERS J.C.
<b>CATEGORY OF CITED DOCUMENTS</b>			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons  & : member of the same patent family, corresponding document	